Explor Anim Med Res,
Vol.10, Issue - 2, 2020, p. 188-194

ISSN 2277- 470X (Print), ISSN 2319-247X (Online)
Website: www.animalmedicalresearch.org

Research Article

SYNTHESIS AND CHARACTERIZATION OF COPPER OXIDE NANOPARTICLES AND THEIR CUTANEOUS WOUND HEALING POTENTIAL

Vijayta Gupta[1], Raju Prasad[2*], Pinki Singh[3], Vinay Kant[4], Pawan Kumar[5], Meena Sharma[1]

Received 17 December 2019, revised 26 November 2020

ABSTRACT: **The remarkable financial burden and social impact due to impaired wound healing is forcing the research towards the development of newer drugs or formulations to accelerate the healing. In present study, effects of topical application of copper oxide (CuO) nanoparticles were evaluated in cutaneous wound healing in rats. One square shaped open excision wound (approximately 400 mm^2) was created on the back of fifteen rats and rats were equally divided into three groups viz. Group I, II and III. Ointment base, bulk CuO (0.3%) and CuO nanoparticles (0.3%) were topically applied on wounds of rats of group I, II and III, respectively for 15 days.The CuO nanoparticles treated group showed significant smaller wound area and increased per cent wound contraction on day 5, 10 and 15 post-wounding in comparison to control group. Histopathologically, wounds treated with CuO nanoparticles has decreased fibroblast number, decreased number of capillaries and compact collagen arranged in well-organized manner and orientation. The regeneration of epithelial layer covering more area of healing tissue was only evident in the CuO nanoparticles treated group. The overall wound maturity score was more evident in the CuO nanoparticles treated group as compared to other groups. In conclusion, topical application of CuO nanoparticles (0.3%) has pronounced healing potential than its bulk form and its applications may be extended to complicated wounds.**

Key words: CuO nanoparticles, Wound healing, Collagen, Epithelial layer, Rats.

INTRODUCTION

Skin serves as a protective barrier against any harmful environmental insult and loss of the integrity of large portions of the skin as a result of injury or illness that may lead to morbidity or even death (Singer and Clark 1999). Loss of integrity of the body structures may occur by physical, chemical, thermal or microbial insult and is called wound. Wound healing is an integral part of recovery of wound (Norris *et al*.1990). Healing is the process by which body restores the injured part to as near its previous normal condition. Once a wound occurs, a multitude of biological and chemical processes are set in motion. Normal wound involves four temporal overlapping phases *i.e*. haemostasis, inflammation, proliferation and remodelling (Donald and Zachary 2004). Wound healing is a burning problem and constitutes an important aspect in rehabilitation medicine. Despite some recent advances in understanding its basic principles, problems in wound healing continue to cause significant morbidity and mortality, particularly in animals (Fine and Mustoe 2006). Cutaneous wounds in addition to causing pain and discomfort and predisposing the patient to superficial and chronic infection, involve significant cost associated with the long-term treatment. The incidences of wounds and complicated wounds in patients are increasing day by day. Wound-healing impairment is characterized by the inability of the healing process to progress, thus, leaving the wound susceptible to external infections as well as deterioration of the underlying tissue, and leading to morbidity and sometimes requires amputation (Wertheimer 2004). Several drugs and formulations have been used and are in use for the

[1]Dept. of Chemistry, University of Jammu. [2]Dept. of Pharmacology and Toxicology, Birsa Agricultural University, Ranchi, [3]Dept. of Zoology, Ranchi University, Ranchi.[4]Dept. of Vety. Pharmacology & Toxicology. [5]Department of Pathology, IVRI, Bareilly, UP. India.
**Corresponding author. e-mail: drrajuprasad@gmail.com*

management of sterile, infected and complicated wounds. Variety of treatment modalities available for wound repair include application of antibiotics, occlusive layers, bandages, poultices and mechanical devices that reduce evaporation of water and others. However, all of these modalities just support the body mechanisms to heal the wound and unfortunately, these become less effective for wound healing process when immunity or other body functions are compromised. To cope up such situations, there are desires for compounds that speed up the healing by actively regenerating the skin, dermis and epidermis.

Different metal has been used from ancient time for the normal functioning of the body. Copper is one of the metals, which is an essential trace element for humans and animals and the ancients recognized copper as an essential healing mineral. Copper is used since several years as a magical element, as it is known to possess anti-microbial, anti-inflammatory, and angiogenic properties used in the treatment of skin ailments, most importantly wound healing (Agarwal *et al*. 2017). It facilitates the activity of several enzymes (Borkow and Gabbay 2009) and provides a role in the development and maintenance of the cardiovascular, skeletal and nervous system. It is evidenced that copper has potent antibacterial properties and is an essential element in many wound-healing-related processes (Borkow and Gabbay 2005). Copper is considered as an excellent active ingredient to be used in products, which come in contact with the skin, aiming to improve the skin's well-being. Copper plays a key role in the synthesis and stabilization of skin proteins, and it also has potent biocidal properties (Borkow *et al.* 2010). In recent years, the emergence of nanotechnology had led to the synthesis of different type of nano formulations of metal and their oxides for the evaluation of different biological activities. Copper oxide (CuO) nanoparticle composites have shown the pronounced antimicrobial activity (Tran *et al*. 2017). Studies have revealed that the biological effects are better observed for nano forms as compared to the bulk form of metal and metal oxides. In view of this, the present study was planned to evaluate the wound healing potential of copper oxide (CuO) nanoparticles in comparison to its bulk form.

MATERIALS AND METHODS

The chemical precipitation method, as described in our previous study, was used for the synthesis of CuO nanoparticles (Gupta *et al.* 2015). Briefly, precipitation of copper salt in alkaline medium was done for the synthesis of CuO nanoparticles. Thereafter, pouring of sodium hydroxide solution was done in copper chloride and glacial acetic acid mixture on magnetic stirrer till the pH of solution reached to 12.5. The formation of black colored suspension occurs, which was later centrifuged to get CuO precipitates. These precipitates were kept at 400°C for 3h to obtain nano CuO. Characterization of synthesized CuO nanoparticles was carried out by using particle size analyzer (PSA) and scanning electron microscopy (SEM). The particle size distribution (PSD) analysis of the synthesized nanoparticles was done by Malvern Instruments Zetasizer Nano-ZS instrument. Scanning electron microscope (JEOL JSM - 6390LV) was used for the determination of surface morphology and confirmation of nanoparticles size.

Preparation of ointment base was done by mixing hard paraffin (5%), soft paraffin (90%) and lanolin (5%). Bulk CuO (0.3%) and CuO nanoparticles (0.3%) were mixed with mixed with ointment base by the incorporation method.

Fifteen healthy male rats (140-160 g) were anesthetized by intraperitoneal (i.p.) injection of ketamine (50 mg/kg, i.p.) + xylazine (5mg/kg, i.p.) combination. Dorsal skin of the anesthetized rats was shaved and cleaned with 70% ethanol. Thereafter a square shaped full thickness excisional cutaneous wound (≈400 mm^2) was created on the back (thoraco-lumbar) region of the rats. Each wounded rat was housed separately in disinfected cages and all rats were divided equally in three groups (five each). *Ad libitum* access to feed and water, 12-hour light-dark cycle and adequate ventilation was provided to rats during the whole experiment and the experimental protocol was approved by the Institute Animal Ethics Committee prior to experimentation. In group I (Control or vehicle or ointment base-treated), ointment base was applied topically once daily for 15 days. In group II (CuO-treated), bulk CuO (0.3%) in ointment base was applied topically once daily for 15 days. In group III (Nano CuO-treated), CuO nanoparticles (0.3%) in ointment base was applied topically once daily for 15 days.

Wound of each rat was photographed on days 0, 5, 10 and 15 post-wounding for the gross evaluation of wound healing. Tracing of each wound margins was done on a transparent paper with the help of fine tip permanent marker and area (in mm^2) within the boundaries of each wound tracing was determined planimetrically. The wound area on 0 day of each animal was measured at a predetermined time interval starting at 3 h post-wounding and subsequent measurements of wound areas from all the groups were taken on day 5, 10 and 15 post-wounding. The result of wound measurements was expressed as absolute values and relative values or per cent wound contraction (Kant *et al.* 2013). The absolute values were

actual measurements of wounds obtained at each given interval, whereas in relative values the wound contraction was expressed as per cent values of the 0-day measurements and was calculated by Wilson' formula as follows:

$$\% \text{ wound contraction} = \frac{\text{0 day wound area} - \text{wound area on particular day}}{\text{0 day wound area}} \times 100$$

After fifteen days of different treatment, the healing/ healed tissue from each rat was collected under general anesthesia (xylazine + ketamine combination). A piece of collected tissue was immediately preserved in 10% neutral buffered formalin for histo-pathological studies.

For Hematoxylin and Eosin (H&E) staining and scoring, the collected tissues fixed in formalin were embedded in paraffin wax and cutting of tissue sections (6 µm thick) was done. The H & E staining of one set of tissue sections were done for each rat of all groups as per standard method. The stained sections were analyzed and scored under a light microscope at different magnifications. Scoring for the inflammatory cells (Hajiaghaalipour *et al.* 2013), epithelialization (Abramov *et al.* 2007) and overall wound maturity (Greenhalgh *et al.* 1990, Kant *et al.* 2015) was done as per the standard methods. Scoring for blood vessel density was done by counting the number of blood vessels in 20 random fields at higher magnification (40X). The histo-pathological properties of different tissue sections and scoring were blindly done. Masson's trichrome staining of each tissue section was done as per standard method for collagen analysis. The stained sections were analyzed under a light microscope at different magnifications. In this staining, nuclei stained black; cytoplasm, muscles and erythrocytes as red and collagen stained blue.

All data were expressed as mean ± standard error of mean (S.E.M.) of five animals. Data were analyzed by one- and two-way analysis of variance (ANOVA) followed by Bonferroni's post-test using the GraphPad Prism v4.03 software program (San Diego, CA, USA). The differences between the different treatment groups were considered statistically significant at $p \leq 0.05$ or lower.

RESULTS AND DISCUSSION

The CuO nanoparticles gave an overall z-average size of 179.2 nm with a polydispersity index of 0.506. The SEM image of the CuO nanoparticles showed rod shaped CuO nanoparticles with the average size of 90-111 nm

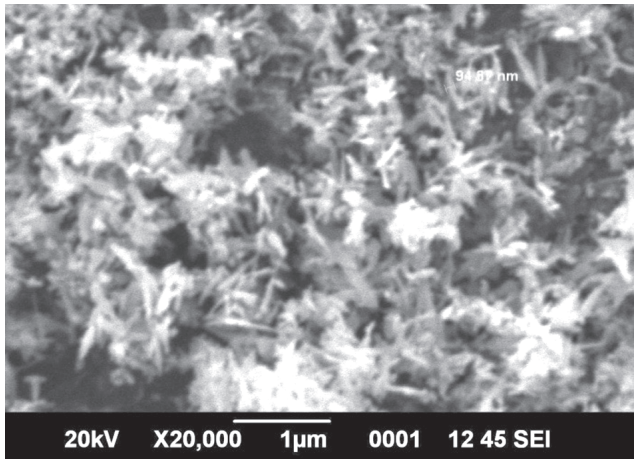

Fig. 1. Representative SEM image of CuO nanoparticles.

(Fig. 1). The representative gross photographs of wounds of different groups on various days were revealing that wound closure was faster in CuO nanoparticles treated group, as compared to other groups (Fig. 2a). The results of wound area depicted that the wound area size decreased in a time-dependent manner in all the groups and CuO nanoparticles treated group has smaller wound area on respective days, as compared to other groups (Fig. 2b). The CuO nanoparticles treated group showed significant smaller wound area on day 5, 10 and 15 post-wounding in comparison to control group. This was further supported by the significant increased per cent wound contraction in CuO nanoparticles treated group on different days, as compared to other groups (Fig. 2c). The bulk CuO treated group also showed some marked reduction in wound area and increased wound contractions on different healing days in comparison to control group. But, the extent of effect was more significant and pronounced in CuO nanoparticles treated group during the whole experiment.

Contraction is an important component of proliferative phase, which involves pulling of surrounding skin circumferentially toward an open wound resulting in wound closure without formation of new tissues (Bae *et al.* 2012). Wound contraction usually began around the day 5 after wounding and was complete by 12-15 days (Peacock 1984). Monocytes and fibroblasts are necessary for normal wound contraction (Karr *et al.*1995). The primary role of the fibroblast in wound healing is the production of extracellular matrix (ECM) composed primarily of collagen and fibronectin. ECM creates the scaffolding on which granulation tissue formation, keratinocyte migration, and wound contraction can be accomplished. Collagen is a major protein of the ECM, which contributes to the wound strength and plays an important role in homeostasis and epithelization (Singer

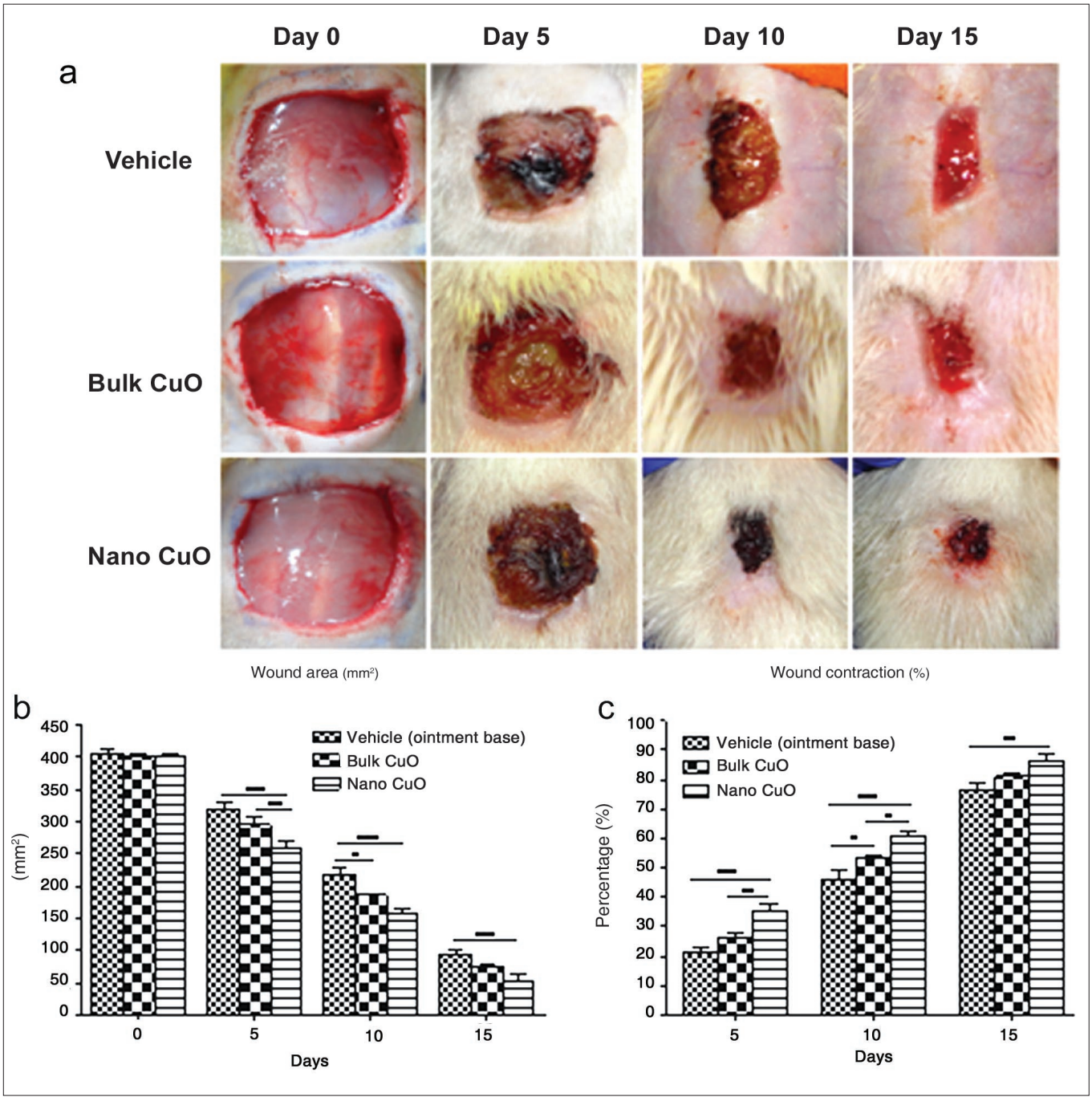

Fig. 2. (a) Gross photographs of wound of different groups on days 0, 5, 10 and 15 post-wounding; (b&c) Effects of topical application of nano CuO on (b) wound area and (c) wound contraction in rats. Data are expressed as Means ± SEM. *, ** and *** represent $p<0.05$, $p<0.01$ and $p<0.001$ (n=5).

and Clark 1999). Increased proliferation and transformation of fibroblasts into myofibroblasts (Tomasek *et al*. 2002) under the influence of TGF-b1 (Desmouliere *et al*. 1993) lead to faster wound contraction. Thus, the increased wound contraction in CuO nanoparticles treated group might be attributed to these cellular changes occurred at wound site.

The quality of healing and status of different cells associated in the healings was confirmed in the present study at the end of experiment by histopathological studies of the healing tissue. The representative pictures of H&E stained, and Masson's Trichrome stained wound sections of different groups are shown in Fig. 3a and Fig. 3b, respectively. The semi-quantitative score of H&E stained sections for inflammatory cells, blood vessels, epithelization and wound maturity is shown in Fig 3c,

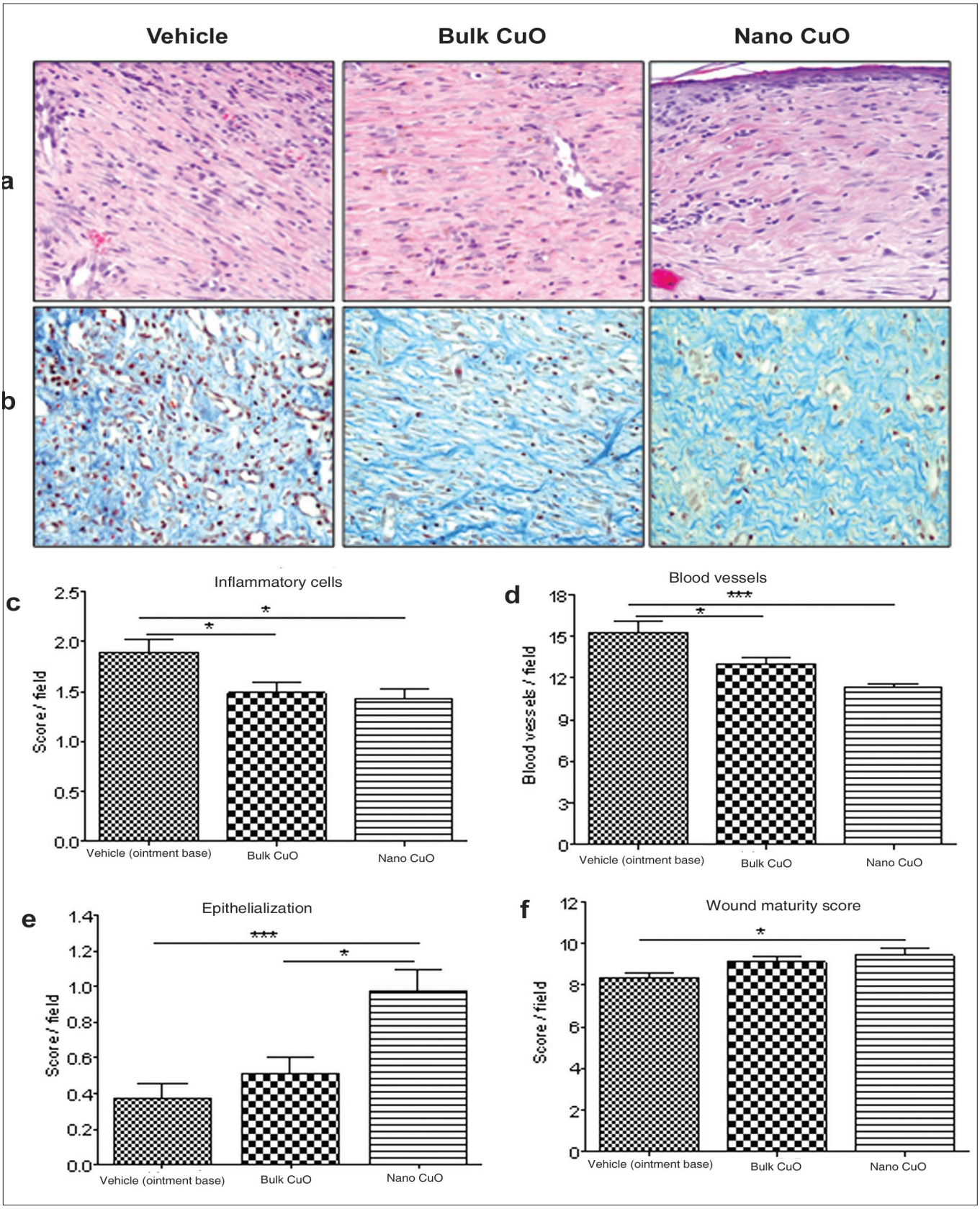

Fig. 3. Representative images of (a) Haematoxylin & Eosin (H & E) (20X) stained and (b) Masson's Trichrome stained wound sections of different treatment groups. Semi-quantitative score of (c) inflammatory cells, (d) blood vessels density, (e) epithelialization and (f) wound maturity in H & E stained cutaneous wound sections of different treatment groups. Data are expressed as Mean ± SEM. * and *** represent $p<0.05$ and $p<0.001$.

3d, 3e and 3f, respectively. It was evident from the H&E stained sections that the wound sections showed well-formed granulation tissue with inflammatory cells, fibroblast, blood vessels and collagen formation. The control group showed the presence of more mixed type inflammatory cells as compared to other groups. Different types of inflammatory cells stimulate positive feedback mechanism to produce additional cytokines and growth factors, which further play vital role in the proliferative phase of wound repair (Werner and Grose 2003). Deposition of large amounts of extracellular matrix occurs after the migration of different cells into the provisional matrix. However, the inflammatory cells should not persist for longer durations as the secretions of different inflammatory cytokines for longer time may decrease the wound healing. In the present study, the inflammatory cells were lesser in the CuO nanoparticles treated group, which revealed that the proper resolution of inflammatory phase in this group and resulted in faster wound healing.

Angiogenesis is an important component of wound healing in which new vessels appear as early as day 3 after wounding (Brem and Folkman 1994) by involving endothelial migration, proliferation, and tube formation. Blood vessels formation during early stage of healing is necessary for delivery of oxygen and nutrients, and removal of waste metabolites for proper healing process. Wound healing gets delayed during impairment of angiogenesis (Brem *et al*. 1997). There are many growth factors known as pro-angiogenic factors, which stimulate the angiogenesis and some identified and well characterized factors include basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), platelet-derived growth factor (PDGF), placental growth factor (PlGF), TGF-β, and VEGF (Johnson and Wilgus 2012). However, there should be apoptosis of blood vessels during the remodeling phase of healing for proper maturity of healing tissue. In present study, numbers of blood vessels were more in control group, as compared to other groups. The fibroblast dominance was also more evident in control group as compared to other groups.

Collagen fibers are considered important components of the extracellular matrix, which provide the tensile strength to the healing wounds (Ono *et al.* 1999). During the earlier stages of wound healing, the dominance of delicate and loosely arranged collagen fibers is observed. However, collagen fibers reveal a well-organized pattern and more compactness in thicker bundles during the later stages of the healing process (Pereira *et al.* 2010). Thus, arrangement and compactness can also be considered as one of the indicators of wound maturity. In present study, the collagen synthesis and deposition was more in bulk CuO and nano CuO treated group as revealed by H&E stained and Masson's Trichrome stained wound sections. However, the collagen fibers were well oriented and compacted in CuO nanoparticles treated group as compared to other groups. This group also showed the wavy pattern of mature collagen fibers, which was lacking in other groups.

Further, re-epithelialization also has vital role in wound contraction and optimal wound healing. The underlying contractile connective tissue shrinks in size to bring the wound margins toward one another and make re-epithelializing easier. In present study, regeneration of epithelial layer covering more area of healing tissue was only evident in the CuO nanoparticles treated group. The other groups showed partial formation of epithelial layer only in few animals and also limited at the margin of wounds. The overall wound maturity score was more evident in the CuO nanoparticles treated group as compared to other groups.

Apoptosis of myofibroblasts, endothelial cells and macrophages, and reduction in the number of capillaries and the amount of ECM occur during the remodeling phase of healing. The histological sections clearly revealed that the wounds treated with CuO nanoparticles had decreased fibroblast number, decreased number of capillaries and compact collagen arranged in well-organized manner and orientation, which are the indicators of entry of the healing process in the remodeling phase in this group. However, fibroblasts dominance, unorganized deposited collagen and presence of a greater number of blood vessels were well evident in control (vehicle treated) group, which revealed that healing process still had not entered in the remodeling phase.

CONCLUSION

In conclusion, topical application of CuO nanoparticles (0.3%) caused faster wound closure and healing process entered earlier in remodeling phase as compared to other groups. The healing potential of nano CuO was better than its bulk form and in future, its evaluation may be extended in complicated wounds of laboratory animals before its applications on clinical wounds.

ACKNOWLEDGEMENT

The authors are thankful and acknowledge the support of SAIF STIC (Kochi), SAIF Chandigarh, CIL Chandigarh for providing the facilities for characterization of nanoparticles.

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***Cite this article as:** Gupta V, Prasad R, Singh P, Kant V, Kumar P, Sharma M (2020) Synthesis and characterization of copper oxide nanoparticles and their cutaneous wound healing potential. Explor Anim Med Res 10(2): 188-194.